

Mutational Analysis of the 18-Base-Pair Inverted Repeat Element at the Bovine Papillomavirus Origin of Replication: Identification of Critical Sequences for E1 Binding and In Vivo Replication

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Replication of bovine papillomavirus requires two viral proteins, E1 and E2-TA. Previously we demonstrated that sequences within an imperfect 18-bp inverted repeat (IR) element were sufficient to confer specific binding of the E1 protein to the origin region (S. E. Holt, G. Schuller, and V. G. Wilson, *J. Virol.* 68:1094–1102, 1994). To identify critical nucleotides for E1 binding and origin function, a series of individual point mutations was constructed at each nucleotide position in the 18-bp IR. Binding of E1 to these point mutations established that both the position of the mutation and the specific nucleotide change were important for the E1-DNA interaction. Equivalent mutations from each half of the IR exhibited similar binding, suggesting that the halves were functionally symmetric for E1 interactions. Each of these mutations was evaluated also for origin function in vivo by a transient-replication assay. No single point mutation eliminated replication capacity completely, though many mutants were severely impaired, demonstrating an important functional contribution for the E1 binding site. Furthermore, E1 binding was not sufficient for replication, as several origin mutants bound E1 well in vitro but replicated poorly in vivo. This suggests that certain nucleotides within the 18-bp IR may be involved in postbinding events necessary for replication initiation. The results with the point mutations suggest that E1-E1 interactions are important for stable complex formation and also indicate that there is some flexibility with regard to formation of a functional E1 replication complex at the origin.

Papillomaviruses have small, double-stranded, circular DNA genomes that replicate episomally in infected cells (7, 9, 15, 16, 38, 54). Productive infection is generally restricted to the natural host, and only a portion of the viral life cycle is recapitulated in cultured cells. Bovine papillomavirus type 1 (BPV-1) has been the prototype for studies of papillomavirus replication (30, 31, 46, 47, 51, 53). The introduction of BPV-1 into mouse cell lines results in a transient amplification of the viral genome followed by persistence at a relatively constant copy number (3, 4, 23, 24, 34). In the culture system, a single origin of replication, which encompasses the unique *HpaI* site on the viral genome (47), has been defined. This same region also functions as an origin of replication in vitro with mouse cell extracts (14, 30, 51, 53). In vivo only two viral proteins, full-length E1 and E2-TA, are required for replication, with the remainder of the replication machinery supplied by the host cell (46). Replication in vitro does not absolutely require either E2-TA or an E2 binding site, though E2-TA greatly enhances replication, especially at low concentrations of E1 (6, 14, 19, 30, 32, 51).

E1 is a multifunctional, nuclear (17, 18) phosphoprotein (5, 37, 42) with origin binding specificity (10, 11, 26, 27, 29, 39–41, 44, 46, 48, 51, 53), helicase activity (40, 52), ATPase activity (28), and the ability to form complexes with the E2-TA protein (5, 25, 29, 44) and the large subunit of DNA polymerase α (32). In the presence of ATP, E1 bound to the origin causes struc-

tural distortions, primarily in the AT-rich region (10). The addition of E2-TA enhances E1 binding to the origin (26, 27, 29, 39, 41, 51, 53), with little or no change in the overall pattern of E1-induced structural changes (10). It has been proposed that E2-TA mediates the initial assembly of E1 at the origin but that the functional replication complex consists of multimeric E1 without bound E2-TA (27). In addition to having a role in mediating E1 assembly at the origin, E2-TA has been shown to bind replication protein A (19) and to overcome nucleosome repression of BPV-1 replication in vitro (20). All of these functions of E2-TA may contribute to its absolute requirement for in vivo replication of BPV-1.

Initial DNA binding studies indicated that the E1 protein, in the absence of E2-TA, could bind to two regions on the viral genome (29, 48, 53). Strong binding was observed in the vicinity of the *HpaI* site, along with weak binding to an additional region approximately 250 bp upstream of the *HpaI* site (48, 53). Subsequent footprinting studies of the *HpaI* region revealed an extended region of protection on both DNA strands (10, 11, 47, 51). Within this extended region was a major protected region between nucleotides 7932 and 15 that encompassed the *HpaI* site (11, 47). Examination of the sequences within the major protected region revealed two alternative, overlapping, imperfect inverted repeats (IR) of 28 and 18 bp, respectively (8, 11, 47, 51, 53). Using double-stranded oligonucleotides corresponding to each IR, we recently demonstrated that sequences within the 18-bp IR were necessary and sufficient to confer E1-specific DNA binding (11). However, formation of a stable E1-DNA complex required the presence of additional sequences beyond the boundaries of the 18-bp IR. Since these flanking sequences were not derived from BPV-1, their functions appear to be sequence nonspecific.

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These data suggest that E1 makes extended contacts with the DNA backbone and that the flanking sequences contribute to overall E1-DNA complex stability.

The previous studies indicated that E1 binding required sequences within the 18-bp IR element, but little is known about the substructure of this region or about specific nucleotides critical for E1 binding and replication function. While several mutations within this region have already been evaluated, these were primarily deletions, insertions, and multiple point mutations (11, 26, 39, 41, 44, 47). Interpretation of these mutations, especially the deletion and insertion mutations, can be difficult, since they affect not only the sequence but also the spacing and relative helical position between potential elements involved in E1 binding and origin function. To evaluate the 18-bp region more systematically, we constructed a large set of individual point mutations throughout this region. Each mutant DNA was tested for *in vitro* E1 binding and for *in vivo* replication. Comparison of the binding and replication properties of these mutants confirmed the importance of sequences within the 18-bp element for E1 binding and origin function, identified specific nucleotides that were critical for replication, and suggested that certain nucleotides contributed to postbinding replication functions.

MATERIALS AND METHODS

Plasmids and phage. Plasmid pBOR was previously designated ORI-105 (11) and contains the 105-bp BPV-1 fragment from nucleotides 7892 to 52. M13BOR contains the same BPV-1 fragment inserted into M13mp18 (50). Plasmid pBOR622 was constructed by ligating a 622-bp *HindIII-Sall* fragment, derived from the pBR322 tetracycline gene region, into pBOR cut with *HindIII* plus *Sall*. The resultant plasmid had the same replication properties as pBOR but was easily distinguished from pBOR on agarose gels because of its larger size. The pBOR622 plasmid was used as an internal standard in transient-replication assays. Plasmids pCGEAG-E1 and pCG-E2 (generous gifts of A. Stenlund) expressed full-length E1 and E2, respectively (46). Plasmid DNAs were purified by cesium chloride gradients or with Qiagen columns (Qiagen, Inc.) and were quantitated by spectrophotometry (optical density at 260 nm). Single-stranded phage DNA was purified by standard procedures (36).

Cell culture. C127 and CHO cell lines were used for electroporation as previously described (8, 46, 47). C127 cells were maintained in Dulbecco minimal essential medium and 10% fetal bovine serum and were used at passages 11 to 15. CHO cells were maintained in Ham's F12 medium with 10% fetal bovine serum.

Mutagenesis of the 18-bp IR region. Point mutations were created by *in vitro* mutagenesis with single-stranded M13BOR DNA and a degenerate oligonucleotide as previously described (50). Phage plaques formed from the mutagenized M13BOR DNA were screened by PCR and single-stranded conformation polymorphism to identify potential mutations and then were confirmed by DNA sequencing with the double-stranded DNA Cycle Sequencing kit (GIBCO BRL). This procedure yielded numerous single point mutations as well as several multiple point mutations and two small deletion mutations. The identified mutations were distributed throughout most of the nucleotide positions of the 18-bp IR region except a few positions within the central *HpaI* recognition sequence. Mutations at these positions were constructed with specific oligonucleotides containing a single mutated base. All single point mutations were designated according to the nucleotide position of their mutation and the nucleotide change.

Preparation of DNA fragments for binding assays. Radiolabeled DNA fragments were prepared by incorporation of [α - 32 P]TTP during PCR amplification (33, 50) of M13BOR and each of the mutant derivatives with primer 1 (5'-TGTGGAATTGTGAGCGGATAAC-3') and primer 2 (5'-TTCCAGTCACGACGTTGTAA-3'). These PCR primers were from the flanking M13 sequences and resulted in amplification products of 256 bp. All the DNA fragments to be generated for a particular DNA binding experiment (see below) were prepared with a common reaction mixture stock and were amplified in a single PCR run so that specific activities would be equivalent; this was confirmed by direct measurement of specific activities. For the ratio binding assay (see below) a radiolabeled 205-bp origin-containing fragment was prepared from M13BOR by PCR incorporation with primer 1 and primer 3 (5'-GACCATGATTACGAATTCGAGC-3'). In addition, a 151-bp fragment lacking the origin was prepared by PCR amplification of pUC18 with primers 1 and 2.

DNA binding assays. Bacterial extracts containing or lacking the RecA-E1 protein were prepared as previously described (48). Binding of RecA-E1 to wild-type and mutant DNA fragments was evaluated by an immunoprecipitation assay as previously described for cloned oligonucleotides (11). Briefly, 10 μ l of bacterial extract was immunoprecipitated with anti-E1 antibody 5996 or preim-

mune serum, and the pellet was washed three times with 1 ml of 10 mM TNE (10 mM Tris-HCl [pH 7.0], 10 mM NaCl, 0.01 mM EDTA) supplemented with 200 mM NaCl, 0.25% Nonidet P-40, and 5 μ g of sheared salmon sperm DNA per ml and was washed one time with 1 ml of 10 mM TNE as described previously (49). These samples were then incubated at 25°C for 30 min with excess radiolabeled PCR-amplified fragments in 150 mM TNE (10 mM Tris-HCl [pH 7.0], 150 mM NaCl, 0.01 mM EDTA). In the direct assay, each sample contained a single species of radiolabeled DNA, either the wild type or one of the mutant fragments, while in the ratio assay, an equal amount of the 205-bp wild-type origin PCR fragment or of the 151-bp nonorigin PCR fragment was included with each 256-bp DNA fragment to serve as an internal standard and competitor. After incubation, the samples were washed three times with 1 ml of 10 mM TNE supplemented with 200 mM NaCl, 0.25% Nonidet P-40, and 5 μ g of sheared salmon sperm DNA. A final wash was done with 1 ml of 10 mM TNE. Washed pellets were incubated with 15 to 60 μ g of proteinase K for 30 min at 55°C, and then the labeled DNA was extracted with TBE sample buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, 10% glycerol, 2.7% xylene cyanol, 2.4% bromophenol blue, 1.5% sodium dodecyl sulfate [SDS]). The extracted DNA was electrophoresed on 8 or 10% Tris-borate polyacrylamide gels. Gels were dried and exposed to X-ray film for autoradiography with an intensifier screen. The relative amount of each bound DNA fragment was quantitated by densitometric analysis of the autoradiographs with the IS-1000 Digital Imaging System (Inno-tech Scientific Corp.). All autoradiographs used for the quantitative analysis had exposures below the saturation level of the film. For each mutation analyzed in the ratio assay, the ratio of bound 256-bp mutant fragment to 205-bp wild-type fragment was normalized to the ratio of a control sample containing both 256-bp and 205-bp wild-type fragments.

Transient DNA replication assays. In the ratio assay (see below), C127 cells (2×10^6 cells per electroporation) were electroporated with 5 μ g of pBOR plasmid (wild type or mutant), 1 μ g of pBOR622 plasmid, 3 to 5 μ g of pCGEAG-E1, and 3 to 5 μ g of pCG-E2. For the direct assay, the pBOR622 DNA was not included. At 8 to 12 h postelectroporation, the cells were washed with phosphate-buffered saline, pH 7.5, and the medium was changed. Cells were maintained 3 to 5 additional days before harvesting.

The electroporation of CHO cells (5×10^6 cells per electroporation) was performed as described previously (8), with the same quantities of plasmids as was used for C127 cells. The medium was also changed as described above, but cells were harvested only 2 to 4 days after the medium was changed. All cells were incubated at 37°C in 5% CO₂ until harvested.

Cells were harvested by the Hirt method as previously described (31) with the following modifications. Briefly, cells were lysed with 800 μ l of lysis buffer (10 mM Tris-HCl [pH 7.6], 20 mM EDTA, 0.6% SDS) for 15 min at room temperature. After lysis, 250 μ l of 5 M NaCl was added, and the lysates were incubated at 4°C overnight. The samples were centrifuged at $12,000 \times g$ for 30 min to pellet cellular debris, and then the lysates were transferred to a sterile tube and digested with 100 μ g of proteinase K per ml for 6 to 12 h. The samples were then extracted with an equal volume of phenol-chloroform, extracted once with chloroform alone, and precipitated with 0.6 to 1 volume of isopropanol at -20°C for 1 to 4 h. The samples were centrifuged at $12,000 \times g$ for 30 min, and the nucleic acid pellets were rinsed with 70% ethanol. Pellets were dried, resuspended in 20 μ l of TE (10 mM Tris-HCl [pH 7.0], 0.01 mM EDTA) with 20 μ g of RNase per ml, and incubated for 20 min at 68°C. The entire 20- μ l sample was digested for 16 h at 37°C with 10 to 100 U of *DpnI* to cleave unreplicated DNA and 10 U of *HindIII* to linearize the plasmids. In parallel control samples consisting of pUC18 DNA or pBOR electroporated without the E1 expression vector, this digestion procedure was sufficient to eliminate all unreplicated input DNA.

Samples were electrophoresed on 1% agarose gels and transferred to nylon by the downward Southern blot method (13). Membranes were baked at 80°C for 30 to 60 min. pBOR DNA was radiolabeled with the Megaprime Random Priming kit (Amersham) as recommended by the manufacturer, and the hybridization (65°C) and subsequent washes were done with the radiolabeled pBOR probe as recommended in the Megaprime kit. Quantitation of hybridization to pBOR (wild type and mutants) and pBOR622 DNA was done with the PhosphorImaging System from Molecular Dynamics. For the direct assay, the amount of replication of each mutant origin plasmid was compared directly with the amount of replication of pBOR run in parallel. In the ratio assay, the amount of replicated mutant plasmids was normalized by the recovery of the internal replication standard (pBOR622) prior to comparison with wild-type pBOR replication. All assays for quantitation were performed three to five times, and the results were averaged.

RESULTS

Creation of BPV-1 origin mutations and experimental design for DNA binding assays. The origin of replication for BPV-1 contains an AT-rich region, an E1 binding region, and an adjacent E2-TA binding site (Fig. 1). The E1 binding region was defined initially by DNase I footprinting and encompasses a dyad symmetry region with alternative, overlapping IR elements of 18 and 28 bp (8, 11, 47, 51, 53). More recently we showed that sequences within an 18-bp IR element are neces-

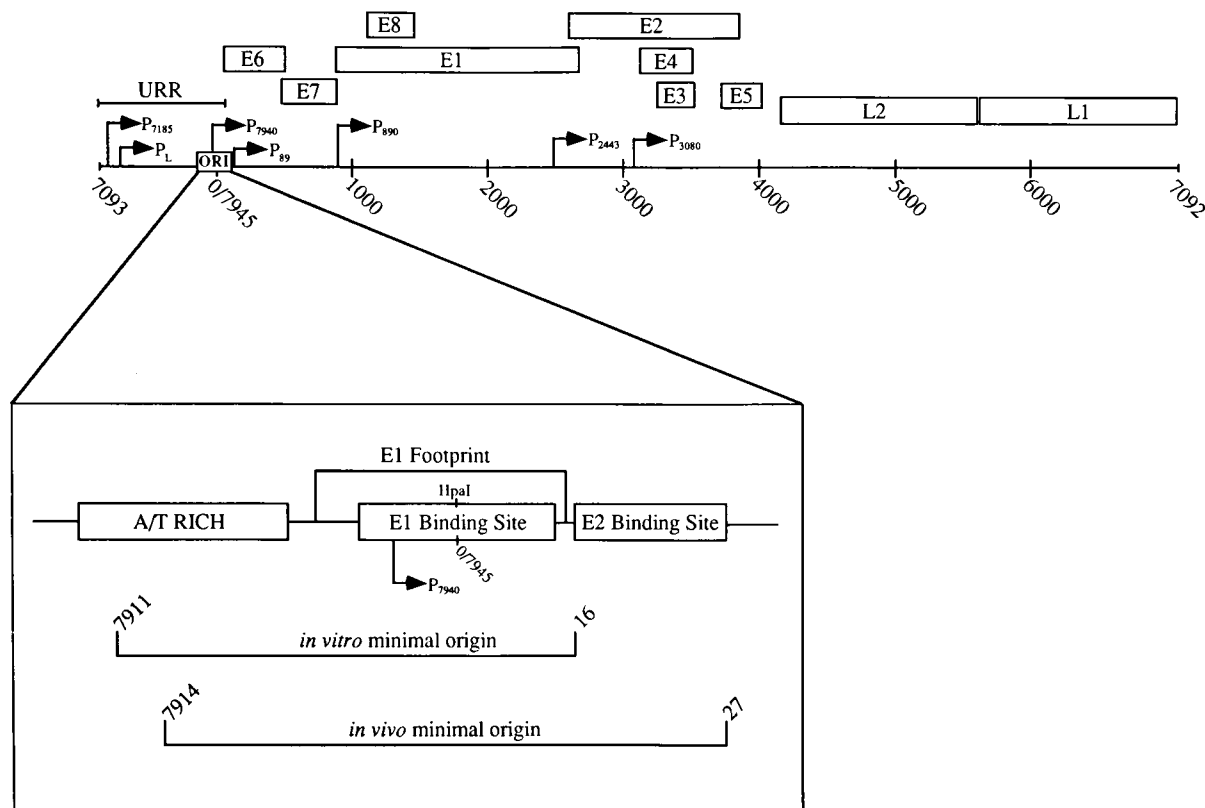


FIG. 1. The bovine papillomavirus type 1 (BPV-1) genome. There are eight early open reading frames (E1 through E8) and two late open reading frames (L1 and L2). Arrows indicate locations of known promoters with the nucleotide positions as indicated. The viral origin of replication (ORI) is located at the unique *HpaI* site within the upstream regulatory region (URR) and is shown in detail in the expanded box. Replication of the virus requires two viral proteins: E1 and E2-TA. The E1 binding site, the E2 binding site, and the AT-rich region represent the minimal BPV-1 origin of replication. Brackets below the origin schematic denote the locations of DNA fragments that function as origins of replication *in vitro* (19) and *in vivo* (45).

sary and sufficient to confer specific binding by E1 protein (11). Neither half of the IR element alone is sufficient for E1 binding (11), but little else is known about the specific sequence requirements.

To develop a more detailed understanding of E1-DNA interactions in the 18-bp IR region, we constructed a large set of single and multiple point mutations throughout this region at each nucleotide position in the element. All of the individual point mutations and two of the double mutations obtained are shown in Fig. 2. For 7 of the 18 positions of the 18-bp element, we isolated two different mutations, while we have isolated only single mutations at the other 11 positions. There are also two mutations, 7933C and 14C, from sequences flanking the 18-bp element. All of these mutations were in the background of M13BOR, which contains a 105-bp fragment of wild-type BPV-1 from nucleotides 7892 to 52. The fragment contains all the *cis* components of the minimal functional origins *in vivo* and *in vitro* (8, 14, 30, 47, 51, 53), as well as additional BPV-1 flanking sequences.

To assess binding of E1 to the various mutant DNAs, uniformly radiolabeled wild-type (256- or 205-bp) and mutant (256-bp) DNA fragments were prepared to equal specific activities by PCR with primer sets from the flanking M13 sequences as described in Materials and Methods. The binding of E1 to each mutant DNA was evaluated by the previously described immunoprecipitation assay (direct assay [11]) or a modified competition form of the assay (ratio assay). For the direct assay, binding reaction mixtures contained a single DNA fragment derived from either the wild-type origin or mutated

origins. In the ratio assay, a 205-bp wild-type DNA fragment was included in each reaction mixture along with the 256-bp fragment being tested. The 205-bp wild-type fragment served as both an internal standard to normalize variation in sample recovery and also as a competitor which enhanced differences between binding of E1 to wild-type and mutant DNAs. For both the direct and ratio assays, the binding reactions were performed in DNA excess as demonstrated by titration experiments (data not shown). Where quantitations are given, each value represents the average of three to six independent experiments.

Binding of E1 to mutated 18-bp IR sequences. A representative ratio binding assay for the single point mutations is shown in Fig. 3. To demonstrate the specificity of the ratio assay, binding was done with a mixture of an origin-containing fragment and a fragment lacking BPV sequences (lanes A to D). Neither fragment was bound in the absence of E1 (lane B) or when preimmune serum was used for the precipitation step (lane C). In the presence of E1 and the anti-E1 serum, only the origin-containing fragment was bound (lane D), indicating that the E1-DNA interaction is highly specific for origin sequences under these binding conditions.

When the ratio assay consisted of two fragments of different overall length but containing identical wild-type BPV-1 origin sequences (Fig. 3, lane WT), there was equivalent binding to both fragments (the ratio of upper to lower bands was approximately 1 after correction for differences in fragment sizes). This is the predicted result for binding conditions under which both DNA substrates are in excess and E1 interacts equally

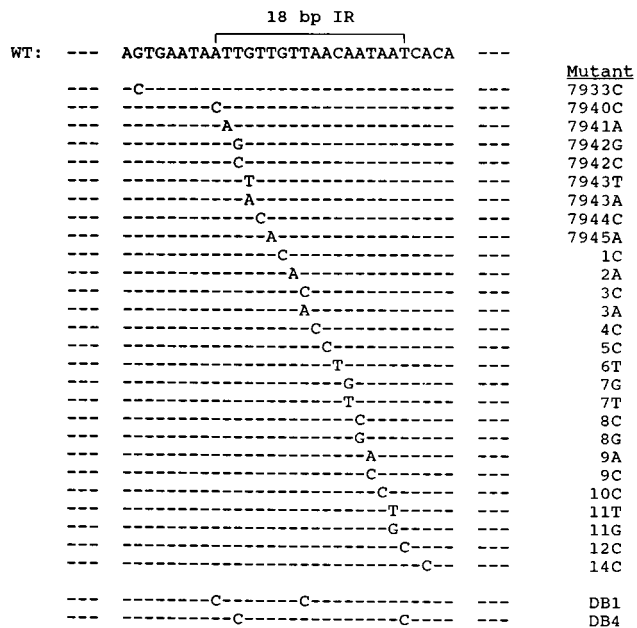


FIG. 2. Wild-type BPV-1 origin segment with corresponding mutations in the E1 binding region. Individual and multiple point mutations spanning the 18-bp IR element were constructed as described in Materials and Methods. The wild-type sequence (WT) is shown at the top, with the subsequent mutated positions shown below. Mutants are designated by their nucleotide numbers and base changes, except for the double mutants, which are designated DB1 and DB4. The position of the 18-bp IR element is indicated by the bracket above the wild-type sequence.

well with each. In contrast, individual point mutations within the 18-bp IR region varied greatly in the relative binding of E1 to the long mutant fragment compared with binding to the short wild-type fragment. Some mutant fragments, such as 7942C, 10C, and 12C, were bound as well as the wild-type fragment was, while others, such as 7943T and 8G, were se-

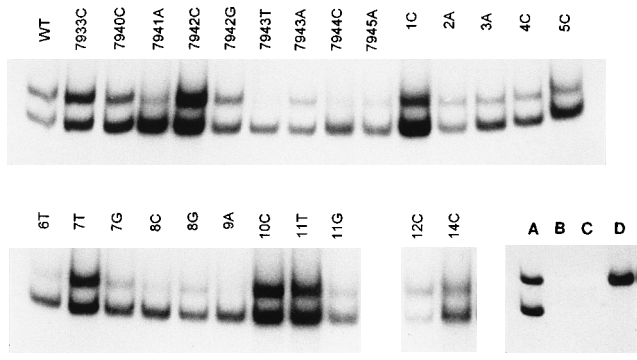


FIG. 3. Representative ratio binding assay. Binding of E1 to the wild-type and mutant DNA fragments was performed by immunoprecipitation as described in Materials and Methods. Shown are the bound DNA fragments that were extracted from the immunoprecipitates, analyzed on a 10% polyacrylamide gel, and visualized by autoradiography. Binding reactions for lanes WT through 14C were performed with extracts containing E1 that had been immunoprecipitated with anti-E1 serum. The lower band in each of these lanes is the 205-bp wild-type fragment included as a recovery control and competitor, while the upper band in each of these lanes is the 256-bp wild-type or mutated fragment as indicated. Binding specificity controls are shown in lanes A-D. Lane A shows the input DNA consisting of the 256-bp wild-type origin fragment (upper band) and a 151-bp pUC fragment lacking BPV-1 origin sequences (lower band). Immunoprecipitations were performed with anti-E1 serum (lanes B and D) or preimmune serum (lane C) with extracts that contained (lanes C and D) or lacked (lane B) E1 protein.

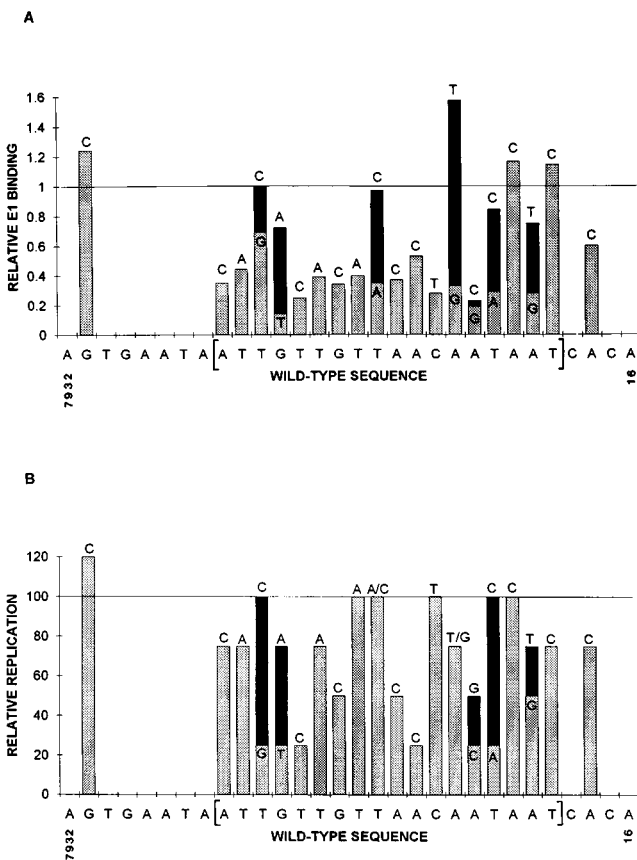


FIG. 4. Summary of binding and replication results with individual point mutations. For both panels, the wild-type origin sequence from nucleotides 7932 to 16 is shown along the x axis. The location of the 18-bp IR element is indicated by brackets. Letters above the bars on the graphs indicate nucleotide changes at those positions. Where two mutations were available at a single nucleotide position, their values are shown by a bar with both shading and black. (A) Shown is the average amount of E1 binding to each mutant DNA relative to that binding to the 205-bp wild-type DNA fragment (ratio assay) (averages are based on a minimum of three experiments; the standard error of the mean was between 0.05 and 0.10 for each sample). (B) Summary of transient DNA replication of origin point mutations. Individual point mutations were tested for in vivo transient DNA replication by the ratio assay as described in Materials and Methods. Each mutant was tested a minimum of three times, and the amount of replicated DNA was quantitated and compared with that of wild-type pBOR. Shown is the average replication for each mutant normalized to wild-type pBOR replication (100%). For graphical purposes, the relative replication levels of the mutations were grouped as follows: (i) from 10 to 25% of wild-type levels graphed as 25, (ii) from 26 to 50% of wild-type levels graphed as 50, (iii) from 51 to 75% of wild-type levels graphed as 75, (iv) and from 76 to 110% of wild-type levels graphed as 100. There was only one mutant outside this range, 7933C, which replicated at 120% of wild-type levels, as shown. Note that all individual point mutants were able to replicate and that none had an efficiency less than 10%.

verely reduced in their binding by E1. The remainder of the mutants displayed a range of E1 binding between these two extremes. In no case, however, did a single point mutation completely eliminate binding by the E1 protein.

Figure 4A shows a quantitative summary of the average relative binding for the single point mutations as determined by the ratio assay. A qualitatively identical pattern was obtained with the direct assay (not shown). Fourteen of twenty-five nucleotide changes within the 18-bp IR element decreased E1 binding to less than 50% compared with that of the wild-type origin sequence. These 14 mutations were located at 13 of 18 positions in the IR element, confirming the importance of this region for E1-DNA complex formation. As expected from our previous results (11), neither of the mutants outside the

18-bp IR region (7933C and 14C) was significantly impaired for E1 binding. However, binding to mutant 14C was consistently reduced by 20 to 40% compared with that of the wild type. This was unexpected, since it was previously shown that nucleotides 13 to 15 could be deleted without affecting E1 binding (26). This slightly reduced binding to the 14C mutant may indicate that the specific nucleotide composition of the flanking sequences can make subtle contributions to overall E1-DNA complex stability.

The 18-bp IR element is an imperfect IR as positions 4 (nucleotide 7943G) and 15 (nucleotide 9T) are not complementary. Two of the mutations generated in this region, 7943A and 9C, restored perfect symmetry to the IR. However, the binding of E1 to these perfect IR elements was slightly reduced compared with that of the wild-type imperfect IR sequence. Clearly there was no enhanced binding, indicating that a perfect IR sequence did not facilitate E1-DNA interactions. The only mutation that consistently gave enhanced binding was at nucleotide 7, where a change from the wild-type A to a T residue resulted in a 50% increase in binding. The significance of the enhanced binding with this particular substitution is unclear.

Examination of the seven positions where two nucleotide substitutions were available revealed different levels of tolerance for change. At position 7942, neither of the nucleotide changes reduced E1 binding significantly, while at position 8 both changes strongly impaired E1 binding. At each of the other five positions (nucleotides 7943, 3, 7, 9, and 11), one mutation had little effect on E1 binding while the other reduced binding by 60 to 90%.

Binding of E1 to double mutants suggests interplay between each half of the 18-bp IR element. In addition to the collection of single point mutations discussed above, the mutagenesis procedure also generated origin clones with multiple point mutations or small deletions. One of the mutations consisted of a single base deletion at nucleotide 7945 in the 5' half of the IR. Binding of E1 to this mutant DNA was less than 20% of that of the wild type (data not shown), which is in agreement with a similar single base deletion mutation described by Spalholz et al. (41). Most of the more complex mutations were extremely impaired for E1 binding (data not shown), and interpretation of their phenotypes was difficult since it was not clear how each of their individual mutations contributed to the binding defect. There were, however, two double mutants, DB1 and DB4, for which each of their mutations was also available as a single mutation (Fig. 5). DB1 contained two mutations in the 5' half of the IR, while DB4 contained one mutation in each half of the IR. For the mutations comprising DB1, 3C alone caused no impairment of E1 binding while 7940C reduced binding by 60%. The binding phenotype of DB1 reflected the defect in 7940C; therefore, having two more severe single mutation. In contrast, neither of the individual mutations comprising DB4 caused any reduction of E1 binding, but DB4 itself was significantly impaired. This suggests that having mutations in both halves of the IR is deleterious even if neither individual mutation affects E1 binding.

In vivo replication of E1 binding site mutants. To compare E1 binding with origin replication function, the wild-type and mutant BPV-1 DNA fragments were subcloned from M13mp18 into a pUC18 background and were assayed for transient DNA replication in vivo. Evaluation in the pUC18 background rather than in the context of the viral genome was chosen because of the transcriptional complexity of the origin region; the functional origin overlaps the P₇₉₄₀ promoter and is just upstream of the P₈₉ promoter (1, 2, 21). In the context of

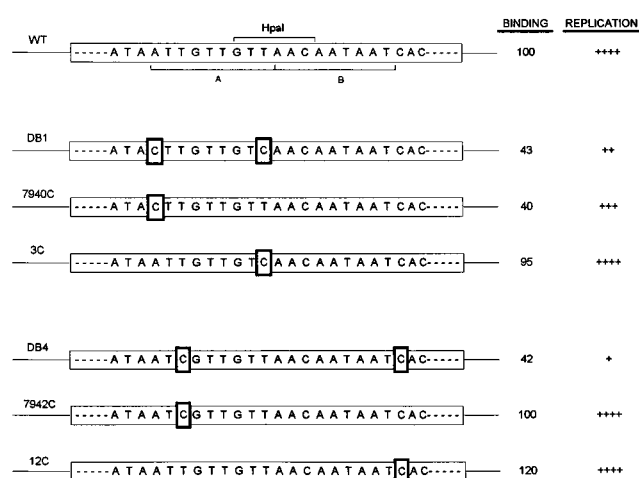


FIG. 5. Binding of E1 to and replication of the double mutants. Shown are BPV-1 origin region sequences for the wild-type clone (WT), two double mutations (DB1 and DB4), and four point mutations (7940C, 3C, 7942C, and 12C). The mutated nucleotides are shown in the boldface rectangles. Above the wild-type sequence, the location of the *Hpa*I site is indicated with a bracket. The 5' (A) and 3' (B) halves of the 18-bp IR are indicated below the wild-type sequence. Binding of E1 to the mutant fragments was assessed with the ratio assay and quantitated as described in Materials and Methods. The numbers in the binding column give the relative binding to the mutant fragment compared with the wild-type fragment and are the averages of at least three experiments. The values in the replication column are the replication levels relative to those of the wild-type origin (++++, 76 to 110%; +++, 51 to 75%; ++, 26 to 50%; +, 10 to 25%). Replication was assayed and quantitated as described in Materials and Methods.

the viral genome, it would be difficult to distinguish between replication and transcriptional effects, since the mutations might be affecting viral gene expression, which could indirectly affect replication competence.

For the direct replication assay, each construct was electroporated into either C127 or CHO cells. The replication signal was stronger in CHO cells, but the relative replication of each mutant origin compared with that of the wild type was unchanged (data not shown). Along with the origin constructs, E1 and E2 expression vectors were coelectroporated to supply the critical viral proteins. For the ratio assay, a fourth construct, pBOR622, was included in each electroporation at a ratio of 1 to 5 with the wild-type or origin mutation construct. pBOR622 consists of the wild-type BPV-1 105-bp origin fragment in a pUC18 background that is 622 bp larger than wild-type pUC18. The pBOR622 construct served as an internal standard for sample handling and recovery during the replication assay.

Figure 6 shows a representative replication assay for pBOR and five of the mutants. As expected, replication in this system was dependent upon both the viral E1 and E2 proteins and a BPV-1 origin. The parental pUC18 vector did not replicate under any condition (data not shown), while pBOR replicated only in the presence of both E1 and E2 (compare lanes 1 and 2). In addition, the amount of *Dpn*I-resistant pBOR product increased with time and was sensitive to *Mbo*I digestion (not shown). All of these features are consistent with authentic BPV-1 origin-dependent replication. In the presence of pBOR622, both pBOR (lower band) and pBOR622 (upper band) replicated at a ratio of 5 to 1, which was consistent with their input ratios. Each of the mutant clones shown, as well as every other single point mutation tested (Fig. 4B), was able to replicate to some degree. However, after normalization of recovery with the pBOR622 band, the mutant origin clones

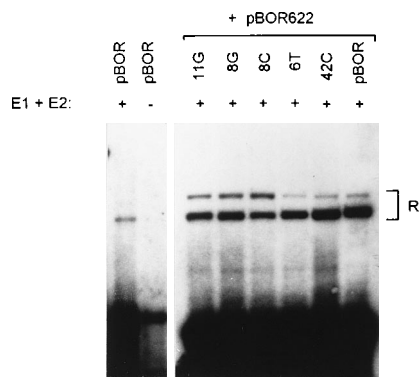


FIG. 6. Representative transient DNA replication of pBOR and origin mutations. Transient replication assays were performed as described in Materials and Methods with C127 cells, and a representative Southern blot is shown. The E1 and E2 expression vectors were included (+) or excluded (–) as indicated. Plasmid pBOR622 was coelectroporated with certain samples as indicated by the bracket above lanes 3 to 8. DNA was harvested at either 3 days (lanes 1 and 2) or 5 days (lanes 3 to 8) postelectroporation and was digested with *DpnI* and *HindIII*. The position of the linear, *DpnI*-insensitive (replicated) DNA is indicated by the bracket and R to the right. In each lane, the upper replicated band corresponds to pBOR622 and the lower band corresponds to the pBOR clone. Designations above lanes 3 to 7 indicate the BPV-1 nucleotide position and the nucleotide change of the mutations being tested. Note that 42C corresponds to BPV-1 nucleotide 7942.

replicated with efficiencies varying from essentially wild-type levels (7942C) to less than 25% of wild-type levels (8C).

Figure 4B shows a quantitative summary of the relative replication levels of the 27 single point mutations examined. Of the 25 single mutations within the 18-bp IR region, 15 gave levels of replication greater than or equal to 50% of that of the wild-type origin, indicating that there was little or no defect in their replication functions. The remaining 10 mutations resulted in more significant replication defects, though all 10 mutants were capable of detectable replication. Neither of the mutations which restored perfect symmetry to the IR element (7943A and 9C) resulted in enhanced origin activity, as both replicated at or just below wild-type levels. Both of the point mutations outside the 18-bp IR sequence (7933C and 14C) replicated at or near wild-type levels, which was consistent with their minimal effects on E1 binding and also indicated that these mutations did not affect the functions of other critical origin elements.

In addition to the single point mutations, the two double mutants, DB1 and DB4, were also assayed for replication (Fig. 5). Both of the point mutations comprising DB1, 7940C and 3C, were located in the 5' half of the IR element. The replication of 3C was essentially wild type, and that of 7940C was somewhat reduced. DB1 replication was similar to that of the mutant with the more severe single mutation, 7940C. The results for DB1 were in contrast to those for DB4, whose component single mutations were located in separate halves of the IR. In this case, both single mutants replicated at wild-type levels, while DB4 was severely impaired and replicated at only 10 to 25% of wild-type levels. Together, the results with DB1 and DB4 suggest that simultaneous damage to both halves of the IR is more debilitating for replication than are two mutations within one half site. A similar conclusion was reached for the effects of these mutations on E1 binding, and it is likely that the replication of these two double mutants directly reflects their relative abilities to be bound by E1.

E1 binding and replication capacity of the single point mutants are consistent with functional symmetry of the 18-bp region. To facilitate analysis of the numerous point mutations,

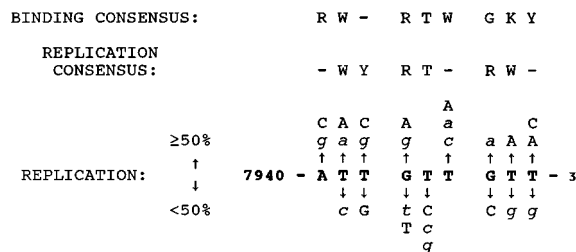


FIG. 7. Summary of replication data and a derived consensus sequence for the 18-bp IR half site. Shown in bold capitals is the wild-type BPV-1 sequence from nucleotides 7940 to 3, which constitutes the 5' half of the 18-bp IR element. Individual nucleotide changes that replicated at $\geq 50\%$ of wild-type levels are shown above the wild-type sequence, while mutations that reduced replication to $< 50\%$ of wild-type levels are shown below. Mutations from both half sites were combined into a single half site for evaluative purposes as discussed in the text. Mutations derived from the 5' and 3' halves of the 18-bp IR are given in uppercase and italicized lowercase letters, respectively. Above the mutations is the consensus sequence for nucleotides critical for replication function. This consensus was derived from the available mutations that replicated at $\geq 50\%$ of wild-type levels. The dash in the consensus sequence indicates that at least three different nucleotides could occupy that position and maintain near wild-type replication. Above the replication consensus sequence is the binding consensus sequence derived in a similar fashion from the binding data in Fig. 4A.

the properties of identical mutations at equivalent positions in each half of the 18-bp region were compared (on the basis of data in Fig. 4). There were five positions where the same mutation was available in each half site: 7941A/11T, 7942G/10C, 7943T/9A, 7944C/8G, and 7945A/7T. With one exception (mutation 7945A versus 7T), equivalent mutations in each half of the IR element exhibited very similar degrees of E1 binding. While the significance of the one exception is unclear, the overall symmetrical trend suggests that the two half sites are functionally equivalent with respect to E1 binding. Likewise, at four of the five positions where identical mutations were available in both half sites, levels of replication were very similar, implying an overall functional symmetry of the half sites. Only at one pair of mutations, 7942G/10C, was there a disparity in replication, with 7942G replicating poorly while 10C replicated at near wild-type levels. The significance of the disparity between 7942G and 10C is unknown but may reflect the intrinsic asymmetry in the organization of the origin region. The E1 binding site is flanked by an AT-rich region on the 5' side and an E2 binding site on the 3' side. The difference in replication for 7942G and 10C may reflect how E1 bound to the 5' and 3' halves of the IR element interacts with the adjacent elements.

Given the apparent functional equivalency of the 5' and 3' halves of the IR for binding and replication, the mutational data for each half site were combined for further analysis. This allowed the evaluation of the effect on binding (data not shown) or replication (Fig. 7) of two or more nucleotide changes at each position in the half site. The effect of a mutation on replication depended on both the position of the mutation and the specific nucleotide change. Three of nine positions in the half site replicated well with any of three nucleotides (positions 7940, 7945, and 3), while only one position failed to replicate well with either available mutation (position 7944). The other five positions retained significant replication with certain nucleotide changes but not with others. On the basis of these levels of replication with different available mutations, a consensus half site sequence of nucleotides critical for replication was derived. A similar analysis of the effect of mutations on E1 binding was performed (not shown), and this analysis defined the consensus half site sequence of nucleotides (Fig. 7) required for efficient E1-DNA interaction. The consensus for replication function was very similar to the

consensus derived for E1 binding, consistent with an essential requirement for E1 binding in order to initiate replication.

DISCUSSION

It has been shown previously that sequences within an 18-bp IR element at the BPV-1 origin of replication are required for binding of E1 protein (11). In the present study, a large collection of point mutations in the 18-bp IR element was created to evaluate the contribution of individual nucleotides to E1-DNA complex formation. Direct binding studies revealed that most of the mutations reduced E1 binding, confirming the importance of this region for specifying E1-DNA interactions. The mutational analysis also revealed a hierarchy of tolerance to nucleotide changes. On the basis of the binding consensus sequence, nucleotide positions 5 and 7 (from left to right in Fig. 7) were intolerant of change; binding was severely reduced with any nucleotide substitution, suggesting that E1 made critical contacts with these nucleotide positions. Position 3 was tolerant of any mutation and, therefore, appeared unimportant for specific E1-DNA interactions. The remaining positions were intermediate in that they could tolerate either of two nucleotides. However, these positions usually required the maintenance of a purine or pyrimidine or of an AT pair.

All of the single point mutations discussed above were only partially defective for E1 binding. Given the symmetry of the E1 binding region, one interpretation of this result is that the 18-bp region represents two adjacent half sites for E1 binding. However, since neither half site alone is sufficient for E1 binding (11), cooperative E1-E1 interactions between molecules bound to each half site must be required for stable protein-DNA complex formation. In this situation, if there is a mutation in only one half of the IR, E1 interaction with the wild-type half may facilitate and stabilize binding of E1 to the mutated half and allow partial binding such as that observed with the single mutations and DB1. Also, this model predicts that simultaneous mutations in both half sites would be more detrimental to binding than would either single mutation alone, which was observed for DB4.

A cooperative binding model is also supported by direct E1-DNA binding curves (26, 39) and studies of E1 assembly at the origin (27). It has been estimated recently that the minimal E1-DNA complex has two molecules of E1 and that a larger oligomeric complex forms that is necessary for DNA replication (27). While it has not been determined in our binding system which type of complex is forming, our data are consistent with each half site of the IR element serving as a binding site for E1 molecules. Whether E1 binds DNA as a monomer and then forms protein-protein contacts while on the DNA has not been determined. However, E1 can form oligomeric structures in solution (12), suggesting that the initial interaction may be between 18-bp IR sequences and dimeric E1. The subsequent recruitment of additional E1 molecules would generate the larger complex observed by Lusky et al. (27) and explain the extended protection seen in footprinting assays (10, 11, 47, 51).

In conjunction with the E1 binding studies, origin mutations were assayed for replication in the context of a 105-bp origin fragment cloned into pUC18, with E1 and E2 proteins supplied in *trans* from heterologous expression vectors. Analysis of the mutations outside of the context of the viral genome avoided any complication caused by pleiotropic effects of the mutations on other viral functions, e.g., transcription, which could indirectly influence replication levels. Therefore, the results obtained in this study are a direct reflection of origin replication function. On the basis of the half site consensus presented in

Fig. 7, only six of the nine positions are critical for replication. Positions 1, 6, and 9 all tolerate at least three different nucleotides without significant impairment of replication. The other six positions are less tolerant and must represent nucleotides required for origin.

For the majority of point mutations (15 of 25), replication levels similar to those of the wild-type origin were obtained even though in vitro E1 binding was significantly impaired for 6 of the 15 mutant DNAs (7940C, 7945A, 2A, 3A, 6T, and 7G). However, it is difficult to correlate in vitro E1 binding capacity with the true affinity of E1 for these six mutant DNAs in vivo. In vivo, each of these mutant DNAs may be capable of adequate interaction with E1 for replication. Additionally, it has previously been shown that full-length E2 protein enhances binding of E1 to wild-type and mutant origins (26, 27, 39, 40). Consequently, it is possible that the presence of E2 in the replication assay overcame an inherently poor binding of E1 to these six mutant origins and allowed normal amounts of replication complexes to form in vivo. In either case, this suggests that none of these six nucleotide changes (or the other nine in this group) affected the ability of E1 to initiate the replication process at the origin once binding had occurred.

The remaining 10 point mutations all exhibited replication levels less than 50% of that of the wild-type origin. While 8 of these 10 mutant DNAs were bound poorly by E1 in vitro, 2 (7942G and 5C) showed moderately good binding. Again, it is possible that in vitro binding of E1 to these mutant DNAs does not accurately reflect the in vivo interaction. However, this implies that the interaction of E1 with all 10 of these DNAs in vivo was so defective that even the presence of E2 protein was insufficient to generate normal levels of replication complexes. A second possibility is that E1 binding alone is not sufficient for DNA replication as has been previously proposed on the basis of other origin mutations (41). In this case the failure to replicate at wild-type levels would be attributed to a defect in some postbinding replicative step rather than to a lack of adequate E1 binding. For example, E1-origin complexes may assemble normally on these mutant DNAs but distortion of origin structure leading to strand separation may be reduced. Further evaluation of the interaction of E1 and E1-E2 complexes with these mutations should help clarify the basis of the replication defects.

In summary, this study has identified functionally critical nucleotides in the origin 18-bp IR element. The general concordance between mutational effects on E1 binding and replication is consistent with E1 binding to this region being necessary for the initiation of BPV-1 replication. This is in contrast to recent observations with some human papillomaviruses that indicate little or no absolute requirement for an E1 binding site (22, 35, 43). Clearly this implies that there may be fundamental differences in how various papillomavirus origins are organized and utilized. More detailed definition of critical elements and their functional interactions is required to understand the spectrum of papillomavirus origins.

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